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A Role of Intracellular Na^+ in the Regulation of Synaptic Transmission and Turnover of the Vesicular Pool in Cultured Hippocampal Cells

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Summary

Propagation of action potentials in axons and dendrites increases intracellular Na^+ ($[Na^+]_i$) and Ca^{2+} concentrations ($[Ca^{2+}]_i$). While the importance of $[Ca^{2+}]_i$ in synaptic transmission is well established, a possible functional role of $[Na^+]_i$ is unclear. In cultured hippocampal cells, $[Na^+]_i$ was increased by veratridine. We have then measured spontaneous excitatory postsynaptic currents (sEPSCs) and, by means of fluorescent dyes, changes in $[Na^+]_i$, in $[Ca^{2+}]_i$, and in the turnover of the vesicular pool of individual boutons. An elevation of $[Na^+]_i$ and a concomitant rise in $[Ca^{2+}]_i$ led to a large increase in sEPSC frequency and in the turnover of the presynaptic vesicular pool. Extracellular Ca^{2+} was essential for these effects of elevated $[Na^+]_i$ on synaptic transmission. They probably occur via Na^+/Ca^{2+} exchange.

Introduction

A vast body of evidence has shown that neurotransmitter release depends on an influx of Ca^{2+} into presynaptic nerve terminals through voltage-gated Ca^{2+} channels (Zucker, 1993; Dunlap et al., 1995; Reuter, 1996). The Ca^{2+} channels are opened during action potentials invading the nerve terminals, thus leading to a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, the main charge carriers responsible for nerve excitation are Na^+ ions. Therefore, during each action potential, there is also a net gain of Na^+ ions in presynaptic nerve terminals. Whether this has a physiological significance for synaptic transmission, however, is not clear. In pinched-off nerve terminals (synaptosomes) from the mammalian brain, an increase in the internal Na^+ concentration ($[Na^+]_i$) leads to an uptake of Ca^{2+} via a Na^+/Ca^{2+} exchanger (Blaustein et al., 1996). Na^+/Ca^{2+} exchange is electrogenic and, depending on its reversal potential, can operate in a forward (Ca^{2+} extrusion) or backward (Ca^{2+} uptake) mode. Uptake of Ca^{2+} into Na^+-loaded synaptosomes, though insufficient to cause neurotransmitter release by itself, has been shown to facilitate the release during subsequent depolarization (Blaustein, 1975; Carvalho et al., 1991). In intact cells, clear evidence that the Na^+/Ca^{2+} exchanger can play a role in synaptic transmission has been provided for chick retinal amacrine cells (Gleason et al., 1994).

Recently, we have shown, by means of a monoclonal antibody directed against the Na^+/Ca^{2+} exchanger, that it is preferentially localized in presynaptic boutons of cultured rat hippocampal cells (Reuter and Porzig, 1995). Similar immunocytochemical evidence has been obtained by Juhaszova et al. (1996). In addition, we could show that the exchanger not only plays a role in reducing $[Ca^{2+}]_i$ in individual presynaptic boutons after electrical stimulation, but can also be involved in the regulation of exocytosis of synaptic vesicles (Reuter and Porzig, 1995). Evidence for a functional role of the Na^+/Ca^{2+} exchanger in Ca^{2+} regulation of intact neurons has been obtained by several other groups (Mulkey and Zucker, 1992; Linden et al., 1993; Kiedrowski et al., 1994; Koch and Barish, 1994).

In the present study, we provide evidence for a powerful role of $[Na^+]_i$ in the regulation of synaptic transmission and in the turnover of the vesicular pool in individual presynaptic boutons of cultured hippocampal cells. An increase in $[Na^+]_i$ by veratridine leads to a rise in $[Ca^{2+}]_i$, most likely via Na^+/Ca^{2+} exchange. This, in turn, causes enhanced transmitter release, measured by spontaneous excitatory postsynaptic currents (sEPSCs). Furthermore, the turnover of the presynaptic vesicular pool in resting cells is enhanced, and the initial rate of exocytosis during electrical stimulation is accelerated under those conditions. This was directly measured by means of the fluorescent styryl dye FM1-43 (Betz et al., 1992; Ryan et al., 1993).

Results

Membrane Potentials and Currents

Figure 1 shows several experimental results that are essential for the interpretation of the main topic of this study: the role of Na^+ in synaptic transmission. Bursts of spontaneous activity could be observed during whole-cell recordings in cultured (days 5–14) pyramidal cell somata (Figures 1A and 1B). They were abolished by tetrodotoxin (TTX; 0.1–1 μM) and reduced by 100 μM Cd^{2+} (data not shown). The resting potential in these cells was -51 ± 4 mV (mean ± SEM; n = 15). Addition of 5 μM veratridine to a Ca^{2+}-containing solution depolarized the cells to -19 ± 3 mV (n = 7), a value similar to that measured in 16 mM KCl solution (-21 ± 3 mV; n = 6). In voltage-clamped cells, veratridine (5 μM) caused its typical effect on I_{Na}, namely a maintained inward current (Figure 1C) that results from long bursts of Na^+ channel openings with a reduced single-channel conductance (Sigel, 1987; Barnes and Hille, 1988). The maintained I_{Na} after repolarization lasted until the drug was washed out. A possible increase in Ca^{2+} channel openings was excluded by measurements of the veratridine effect on total I_{Ba} flowing through these channels (Figure 1D). The alkaloid (5 μM) inhibited peak I_{Ba} by 16% ± 3% and the current at 200 ms by 38% ± 8% (n = 6), indicating a block of Ca^{2+} channels. With 10 μM veratridine, the effect was more pronounced (34% ± 5% and 85% ± 8% inhibition, respectively). The inhibition of I_{Ba} was at least partially reversible during washout of the drug. Cd^{2+} (100 μM) completely and reversibly blocked I_{Ba} (Figure 1E).

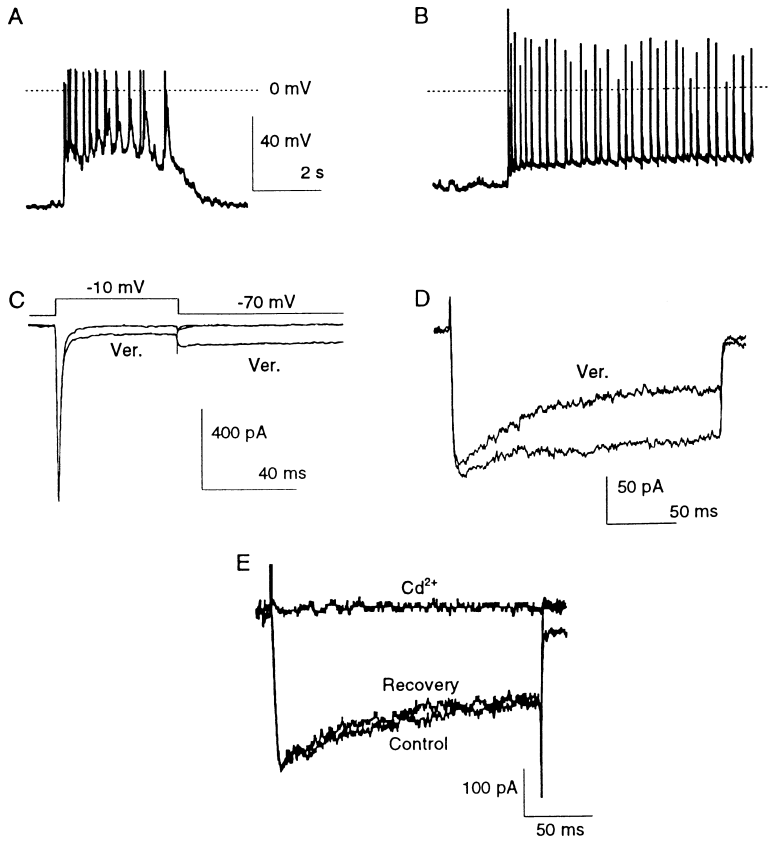

Figure 1. Electrophysiological Recordings in Cultured Hippocampal Cells

(A and B) Patterns of spontaneous bursts of action potentials.

(C) Effect of veratridine (ver., 5 μM) on I_{Na}; note steady inward Na^+ current during and after depolarization.

(D) Veratridine (5 μM) inhibits I_{Ba} through Ca^{2+} channels; note accelerated inactivation.

(E) Cd^{2+} (100 μM) completely and reversibly blocks I_{Ba} through Ca^{2+} channels.

sEPSCs

sEPSCs, presumably resulting from the release of quanta of glutamate from individual presynaptic vesicles, were measured at a holding potential (V_H) of −50 mV. Representative examples of whole-cell current recordings are shown in Figure 2. The observed rapid sEPSCs could be completely inhibited by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), an inhibitor of glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors. This drug also prevented spontaneous excitatory activity of the cultured cells. After blockade of the rapid sEPSCs by CNQX, smaller and slower inward current fluctuations were observed in Mg^{2+}-free solutions. They could be blocked by R(−)2-amino-5-phosphonopentanoic acid (D-AP5; 100 μM), an N-methyl-D-aspartate (NMDA) receptor inhibitor (data not shown; Bekkers and Stevens, 1989). At more positive holding potentials (−20 mV), miniature outward currents could be observed that were blocked by the γ-aminobutyric acid type A ($GABA_A$) receptor blocker bicuculline (10 μM; data not shown).

Figure 2A shows the effect of veratridine on sEPSCs after about 2 min of exposure to the drug. In this example, Cd^{2+} (100 μM) had been added to the solution to inhibit Ca^{2+} currents. Cd^{2+} had no effect on sEPSCs in the absence or presence of veratridine. In two other experiments, a cocktail of the Ca^{2+} channel blockers ω-conotoxin GVIA (5 μM), ω-agatoxin IVA (200 nM), and isradipine (2 μM) was used that also fully blocked I_{Ba} (V_H −50 mV), but had no effect on sEPSC frequencies (data not shown). Veratridine (5 μM) caused a large increase in sEPSC frequency and a shift in the holding current at −50 mV (from −45 pA to −113 pA in Figure 2A). The latter effect is due to the maintained I_{Na} (Figure 1C). Addition of TTX (200 nM) to the solution reversed both effects of veratridine. Histograms of the amplitude of sEPSCs in the absence and presence of veratridine are plotted in Figure 2B. The data were obtained from the experiment in Figure 2A during a 26 s sampling time of sEPSCs under each condition. The number of events during this time was 97 in the controls and 460 in the presence of veratridine. Mean amplitudes of sEPSCs were 38 ± 30 pA (mean ± SD, controls) and 41 ± 25 pA (veratridine), and sEPSC rise times (inset of Figure 2B) were 0.78 ± 0.36 ms and 0.82 ± 0.39 ms, respectively. As known from other studies (for reviews, see Stevens, 1993; Edwards, 1995), the distribution is not normal, but positively skewed. Similar results, with or without Cd^{2+} in the solution, were obtained in 25 cells. Ouabain (50 μM), an inhibitor of the Na^+ pump, potentiated the veratridine (1 μM) effect on sEPSC frequency about 4-fold (Figure 3A). These results strongly suggest that a net gain in $[Na^+]_i$ can increase sEPSC frequencies.

Although the results described above clearly show that the increase in sEPSC frequency by veratridine was independent of Ca^{2+} channel openings, the question remained whether it was dependent on the presence of an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Therefore, measurements were made in Ca^{2+}-free solution. No increase in sEPSC frequency was observed under this condition (Figure 2C), although the holding current was shifted by −140 pA, due to the maintained increase in inward Na^+ current by the drug. During 10 s sampling times in the experiment illustrated in Figure 2C, 258

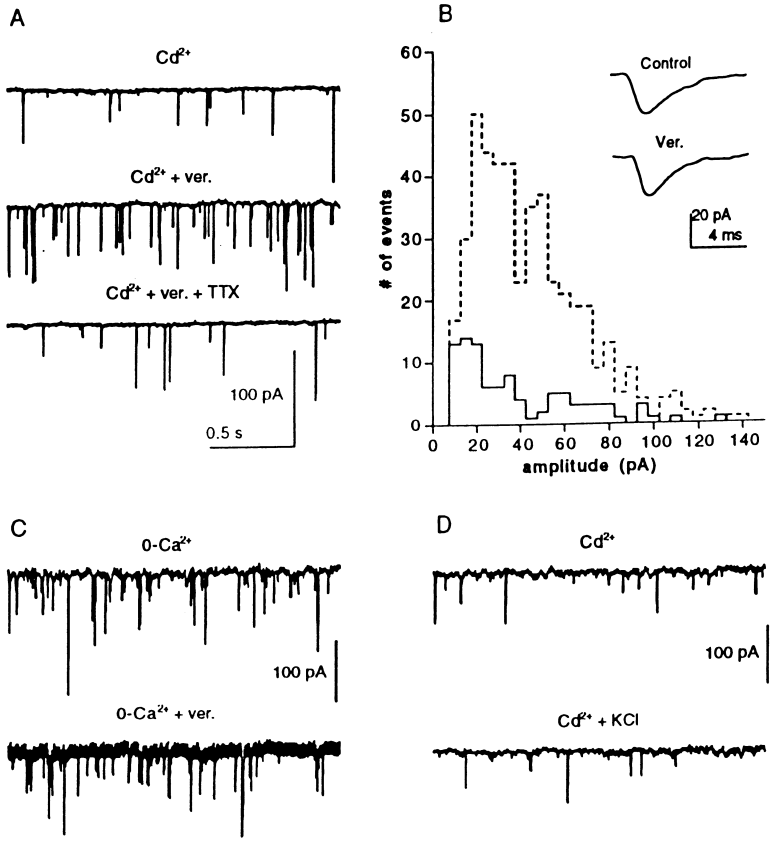

Figure 2. Veratridine Increases sEPSC Frequency Only in the Presence of $[Ca^{2+}]_o$

(A) sEPSCs in Cd^{2+} (100 μM) containing saline (upper row), in the presence of veratridine (ver., 5 μM; center row), and after additional TTX (200 nM; lower row).

(B) sEPSC frequency versus amplitude histograms (bin size 5 pA); sEPSCs were sampled during 26 s each in the absence (full line) and presence (dashed line) of veratridine (5 μM); inset shows representative sEPSCs under both conditions; same experiment as in (A).

(C) sEPSCs in the absence of $[Ca^{2+}]_o$ without (upper row) and with (lower row) veratridine (5 μM).

(D) sEPSCs in Cd^{2+} (100 μM) containing saline with 5 mM KCl (upper row) and 16 mM KCl (lower row). All recordings were made at V_H −50 mV.

sEPSCs were recorded under control conditions and 254 in the presence of veratridine; mean current amplitudes (± SD) were 34 ± 38 pA and 34 ± 54 pA, respectively. These results provide evidence for a dependence on $[Ca^{2+}]_o$ of the veratridine-induced enhanced sEPSC frequency. Similar results were obtained in six experiments: three in nominally Ca^{2+}-free solution and three with additional EGTA (50 μM). Our results are in contrast with those of Nordmann and Stuenkel (1991) in isolated nerve endings from rat neurohypophyses. These authors found a Na^+-dependent, but Ca^{2+}-independent release of vasopressin from these nerve endings. Whether this may indicate that neurotransmitter release and neurosecretion are regulated differently by $[Na^+]_i$ remains to be seen.

Since the Na^+ permeability of the membrane is increased by veratridine, this leads to depolarization and, as will be shown later in the text, to a net gain in $[Na^+]_i$. For the interpretation of our results, these two effects of the drug had to be separated. Therefore, we also

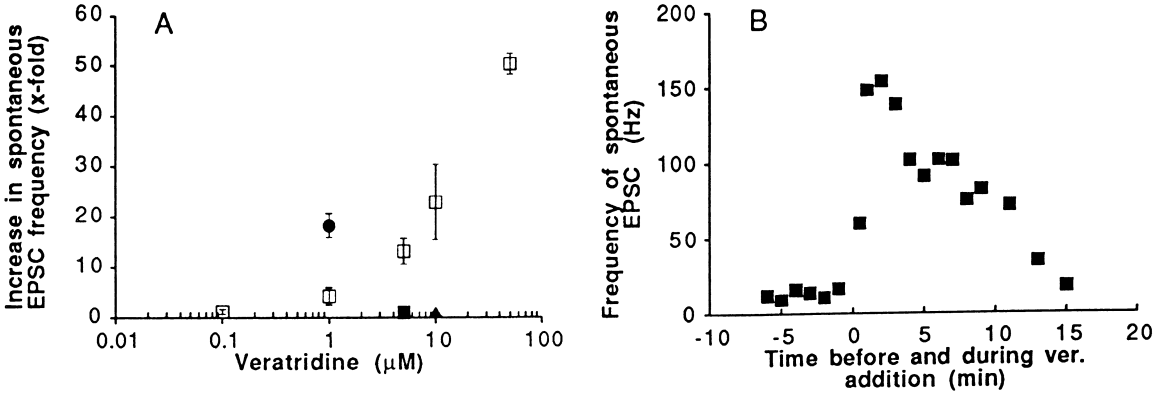

Figure 3. Concentration- and Time-Dependencies of the Veratridine Effect on sEPSC Frequency

(A) Concentration–response relationship of veratridine on sEPSC frequency (open squares); potentiation of the veratridine effect at 1 μM by ouabain (50 μM; closed circle), and inhibition of the veratridine effect at 5 μM by the absence of $[Ca^{2+}]_o$ (closed square), or at 10 μM by TTX (2 μM; closed triangle); means ± SEM of 3–7 cells.

(B) Time course of the veratridine (5 μM) effect on sEPSC frequency; veratridine was added at time 0.

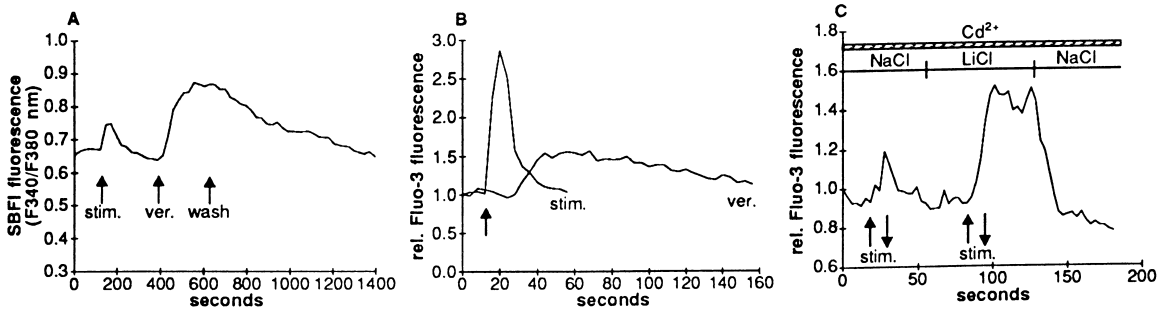

Figure 4. Veratridine Increases $[Na^+]_i$ and $[Ca^{2+}]_i$ in Dendrites

Cultured hippocampal cells (days 18–21) were incubated in SBFI-AM (15 μM) for up to 4 hr and in fluo-3-AM (10 μM) for up to 1 hr.

(A) Fluorescence changes of the Na^+ indicator SBFI in a network of dendrites during electrical stimulation (stim.), during veratridine (ver., 5 μM), and during its washout (wash); the trace is an average of four ROIs in the same culture dish; sampling rate is 0.33 Hz; for details, see Experimental Procedures.

(B) Fluorescence changes of the Ca^{2+} indicator fluo-3 in small areas (~4 μm^2) of dendrites during electrical stimulation (stim.) in the absence of Cd^{2+} or by veratridine (ver., 5 μM; 100 μM Cd^{2+}); the traces are averages of five ROIs in the same culture and were normalized to the fluorescence before stimulation or veratridine application (indicated by the arrow).

(C) Cd^{2+} does not inhibit Na^+-dependent decrease of $[Ca^{2+}]_i$. Fluo-3 fluorescence changes were measured in Cd^{2+} (100 μM) containing NaCl or LiCl salines with 10 mM $CaCl_2$. Brief periods of stimulation (stim.) produced only a small, transient increase in $[Ca^{2+}]_i$ in NaCl solution and a maintained larger rise in LiCl solution; note the rapid fall of $[Ca^{2+}]_i$ after readdition of NaCl solution. The trace is an average of three ROIs in the same culture.

studied sEPSC frequencies in the presence of 16 mM KCl in the solution. As mentioned above, the high K^+ solution produced a depolarization similar to veratridine. However, because K^+ activates the Na^+ pump (Nakao and Gadsby, 1989), a reduction in $[Na^+]_i$ rather than an increase is expected. The experiments were done in the presence of Cd^{2+} (100 μM) to inhibit Ca^{2+} channel currents. No increase in sEPSC frequency was seen under this condition. In the experiment shown in Figure 2D, during a 6.5 s sampling period, 25 sEPSCs occurred under control conditions and 27 in the high K^+ solution. In five similar experiments with high KCl, no significant increase in sEPSC frequency was measured. Therefore, we conclude that the veratridine-induced increase in sEPSC frequency depends on the load of $[Na^+]_i$ and not on depolarization.

Our results with veratridine on sEPSCs are summarized in Figure 3. Veratridine increased sEPSC frequencies 1.2- to 50-fold over a concentration range of 0.1–50 μM (Figure 3A, open squares). In the absence of $[Ca^{2+}]_o$ (six experiments), no veratridine effect could be seen (closed square in Figure 3A; see also Figure 2C). While 50 μM ouabain in the presence of $[Ca^{2+}]_o$ enhanced the veratridine effect (closed circle; three experiments), TTX inhibited it (closed triangle; three experiments). Ouabain by itself increased sEPSC frequency 12.3 ± 3.2 fold. It also increases $[Ca^{2+}]_i$ in presynaptic boutons (Reuter and Porzig, 1995). Long-term recordings, such as that shown in Figure 3B, showed that the veratridine effect on sEPSC frequency reached its peak within 2 min, and then slowly decayed. The rate of decay was greatly reduced by ouabain (three experiments; data not shown), suggesting that a reduction of $[Na^+]_i$ by the Na^+ pump is involved in the decay. The holding current remained constant during this time course, indicating that the decay is not due to run-down of the cell. We conclude that sEPSC frequencies increase when $[Na^+]_i$ is elevated and that this effect depends on $[Ca^{2+}]_o$.

$[Na^+]_i$ and $[Ca^{2+}]_i$ Measurements

To demonstrate directly an increase of $[Na^+]_i$ and $[Ca^{2+}]_i$ by veratridine, we measured changes in these ion concentrations by means of fluorescent dyes. In one set of experiments, we measured $[Na^+]_i$ changes in dendrites by using the membrane-permeable, Na^+-dependent fluorescent dye SBFI-AM (15 μM). An increase in the ratio of SBFI fluorescence emission from excitation at 340/380 nm indicated a rise in $[Na^+]_i$. Since these measurements could not be done by confocal microscopy, rather densely packed areas of dendrites were chosen. The imaging system (Reber and Reuter, 1991) used for these measurements allowed us to identify dendrites clearly, while boutons could not be resolved. Four regions of interest (ROI) were selected within a field of view with no visible glia cells underneath. Within these regions, fluorescence changes in dendrites by electrical stimulation or by veratridine could be seen and faithfully measured. The effectiveness of field stimulation (20 Hz, 1 ms pulse duration, ~10 V/cm electric field) was confirmed by action potential recordings in separate experiments. When the cells were electrically stimulated for 10 s, the resulting action potentials increased $[Na^+]_i$ only slightly (Figure 4A). The signal may have been blunted, however, because of the slow acquisition rate of 3 s per image. The subsequent veratridine (5 μM) application to quiescent cells produced a slower reversible rise in $[Na^+]_i$. In several ROIs, the $[Na^+]_i$-dependent fluorescence began to fall before the washout of veratridine. Similar results were obtained in three other experiments.

Changes in $[Ca^{2+}]_i$ were measured by confocal microscopy as increases in fluo-3 fluorescence. For comparison with $[Na^+]_i$ measurements, areas (~4 μm^2) of a network of dendrites were selected. Ca^{2+} channels were blocked by either Cd^{2+} (100 μM) or by a cocktail of Ca^{2+} channels blockers (5 μM ω-conotoxin GVIA, 1 μM ω-agatoxin IVA, 2 μM isradipine). Figure 4B illustrates such an experiment in cells that had been exposed to

fluo-3-AM (10 μM) for 60 min. During a 10 s period of stimulation (20 Hz) in the absence of blockers, fluo-3 fluorescence increased about 3-fold (averages of seven dendritic sites). Application of veratridine (5 μM) in the presence of Cd^{2+}, after a delay of approximately 10 s, produced a 1.7-fold increase in fluo-3 fluorescence that slowly decayed. This decrease of $[Ca^{2+}]_i$, together with that of $[Na^+]_i$, may be the reason for the slow decay of sEPSC frequency in Figure 3B. The increase in fluo-3 fluorescence during veratridine exposure could be completely suppressed by TTX (1 μM), indicating that the opening of Na^+ channels and, hence, the increase in $[Na^+]_i$ were essential for this effect. Similar results were observed in four other experiments. A possible explanation for these results may be a $[Na^+]_i$-dependent Ca^{2+} uptake via the Na^+/Ca^{2+} exchanger (Reuter and Porzig, 1995). Since Cd^{2+} has been used in many of our experiments to block Ca^{2+} channels, we tested whether it also blocks Na^+/Ca^{2+} exchange. As illustrated in Figure 4C, the presence of Cd^{2+} (100 μM) in the solution had no effect on Na^+-dependent Ca^{2+} reduction. The experiment was done with 10 mM $[Ca^{2+}]_o$ to obtain a large rise in $[Ca^{2+}]_i$ when NaCl was replaced by LiCl. A brief period of electrical stimulation (20 Hz) in NaCl solution produced only a small, transient rise in $[Ca^{2+}]_i$. A much bigger and maintained increase in $[Ca^{2+}]_i$ was seen during a similar period of stimulation in LiCl solution. $[Ca^{2+}]_i$, however, decreased rapidly when the LiCl solution was switched to a NaCl solution. Similar results were obtained in three other experiments. They indicate that Cd^{2+} (100 μM) does not block Na^+/Ca^{2+} exchange in our cells.

We also studied possible intracellular pH changes by means of the membrane-permeable indicator BCECF-AM (10 μM), but could only detect a small acidification with veratridine concentrations higher than 15 μM (data not shown).

Synaptic Vesicle Turnover

The fluorescent dye FM1-43 has been used to measure quantitatively the effect of a Na^+ load on the turnover of the vesicular pool in individual presynaptic boutons. As originally suggested by Betz and colleagues (Betz et al., 1992), this dye can be trapped in synaptic vesicles during endocytosis and is released during exocytosis (Ryan et al., 1993; Betz et al., 1996). Intracellular Na^+ loading was achieved by means of veratridine, as described above. In all experiments, the solution contained CNQX (10 μM) to inhibit recurrent excitatory activity. Figure 5A shows an experiment where vesicles in presynaptic boutons were labeled with FM1-43 (15 μM) during a 30 s stimulation period (20 Hz) followed by a 90 s uptake period. This protocol caused maximal dye loading in presynaptic boutons (Ryan and Smith, 1995). After several minutes washing in dye-free solution to reduce background staining, the label in the boutons remained rather stable under resting conditions (control; normal saline) and in a 16 mM KCl, Cd^{2+} (100 μM) containing solution (left frame). Exposure to 5 μM veratridine (plus 100 μM Cd^{2+}) caused a partial release of the dye as indicated by the dimming of the puncta (center frame). After washout of veratridine and Cd^{2+}, and after a subsequent second loading with FM1-43 under the same conditions as described above, the dye was maximally released from the boutons during a 80 s period of electrical stimulation (20 Hz) (right frame). The time courses of the fluorescence changes in 24 identical boutons under each of these conditions are shown in Figure 5B. Under control conditions and in the presence of KCl and Cd^{2+}, the fluorescence decreased by only 5% and 3%, respectively. The arrow indicates the time of veratridine application and, after the second dye loading, the beginning of the electrical stimulation period. After a delay of about 20 s, veratridine released about 50% of the dye that could be maximally released during the stimulation period. A statistical analysis of the dye release from 56 boutons during electrical stimulation and from 45 boutons after veratridine application is shown in Figure 6B (open columns). After a full FM1-43 load, the difference between maximal release during electrical stimulation and a 2 min period of exposure to veratridine (5 μM; resting cells) is highly significant ($p < 0.0001$). On the average, veratridine released 56% of the dye of the fully labeled vesicular pool during a 2 min exposure. Similar experiments were also done in the absence of $[Ca^{2+}]_o$ (Figure 5C). The cells were loaded with FM1-43 as in Figures 5A and 5B. After washout of nontrapped dye, the cells were superfused for 2 min with Ca^{2+}-free solution containing 50 μM EGTA. Very little dye was lost from boutons of nonstimulated cells during a subsequent 90 s scanning period (ctrl. in Figure 5C). Veratridine (ver. in Figure 5C, 5 μM) was added (arrow) during another scanning period in Ca^{2+}-free solution but, in contrast with Ca^{2+}-containing solution, it only caused a very small reduction in fluorescence. After washout of the drug in normal Ca^{2+}-containing solution, the cells were electrically stimulated (stim. in Figure 5C, 20 Hz; arrow) during a third scanning period. This produced the usual rapid release of the dye from the boutons. The curves in Figure 5C represent averages from 15 identical boutons. The statistical analysis of this and another experiment (30 boutons total) is shown in Figure 6B as dotted columns. In contrast with Ca^{2+}-containing solution, only 8% ± 1.7% of the fluorescence of the fully labeled pool was released during a 2 min exposure to veratridine in Ca^{2+}-free solution. This corresponds to the lack of the veratridine effect on the sEPSC frequency in the absence of $[Ca^{2+}]_o$ (Figure 2C).

In another series of experiments, we have measured the uptake of FM1-43 (15 μM) into presynaptic boutons under the following conditions: resting cells (2 min dye exposure and subsequent extensive rinsing); resting cells plus veratridine (5 μM; same condition as above); electrically stimulated cells (30s at 20 Hz and 90s rest, then washout of the nontrapped dye). The amount of FM1-43 that was taken up into the same presynaptic boutons under each of those conditions was measured by the fluorescence change during subsequent electrical stimulation (20 Hz) and is called "releasable dye." Figure 6A illustrates such an experiment. The curves are averages of 12 boutons. After the loading conditions described above, the time courses of releasable dye during electrical stimulation are shown. In resting cells, dye uptake into a releasable vesicular pool could be

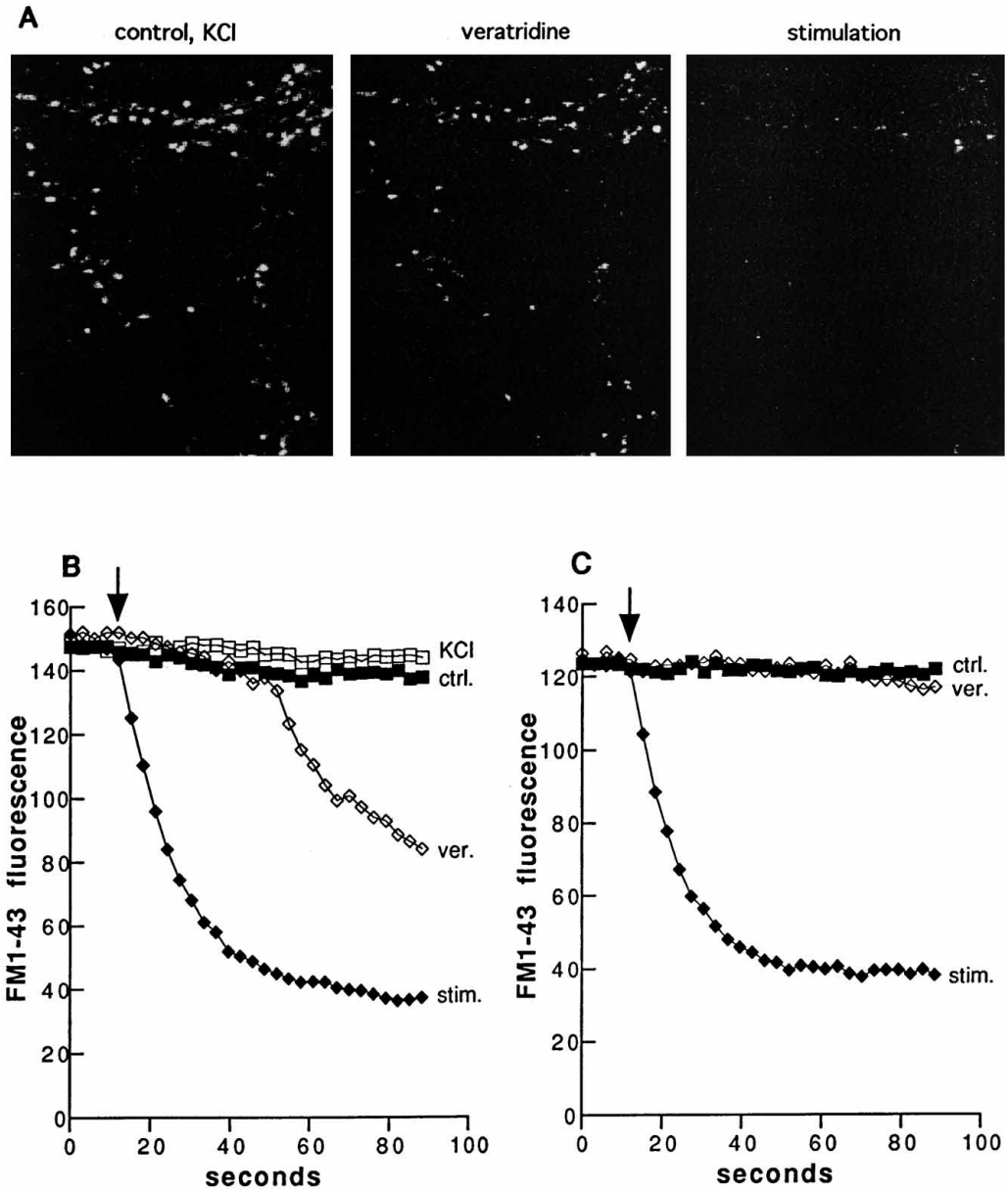

Figure 5. Veratridine Spontaneously Releases FM1-43 from Presynaptic Boutons Only in the Presence of $[Ca^{2+}]_o$

(A) FM1-43 fluorescence in boutons of dendrites after loading (20 Hz stimulation for 30 s and 90 s rest; afterward washout of nontrapped dye for several minutes); fluorescent puncta in normal saline (control) and after addition of high KCl saline (plus 100 μM Cd^{2+}) were identical (left frame); dimming of fluorescent puncta occurred after addition of veratridine (5 μM; center frame); after reloading, a subsequent period of electrical stimulation (20 Hz) caused extensive unloading of the dye (right frame).

(B) Time courses of FM1-43 fluorescence changes under the conditions described above; (closed squares, controls; open squares, KCl depolarization (in the presence of Cd^{2+}); open diamonds, veratridine; closed diamonds, electrical stimulation); averages of 24 identical boutons; the arrow indicates the beginning of veratridine (ver.) application or electrical stimulation (stim.).

(C) Time courses of FM1-43 fluorescence changes in the absence of $[Ca^{2+}]_o$ (plus 50 μM EGTA) under control conditions (closed squares), in the presence of veratridine (open diamonds; arrow) and, in Ca^{2+} containing solution, during electrical stimulation (closed diamonds; arrow); averages of 15 identical boutons.

measured in 7 out of the 12 boutons (Figure 6A, closed squares; rest 1), while in 5 boutons no releasable dye was taken up (Figure 6A, open squares; rest 2). The small linear decrease in fluorescence between the beginning and the end of the scanning period in "rest 2 boutons" is presumably due to bleaching of the dye. A similar trend is seen in later parts of the other traces in Figure 6A. During the following staining period in the presence of veratridine, all 12 boutons at rest took up releasable dye (open diamonds). The amount of dye uptake into the 12 boutons during and after electrical stimulation (full load) is indicated by the filled diamonds. The remaining fluorescence at the end of the dye unloading periods in Figure 6A was background staining

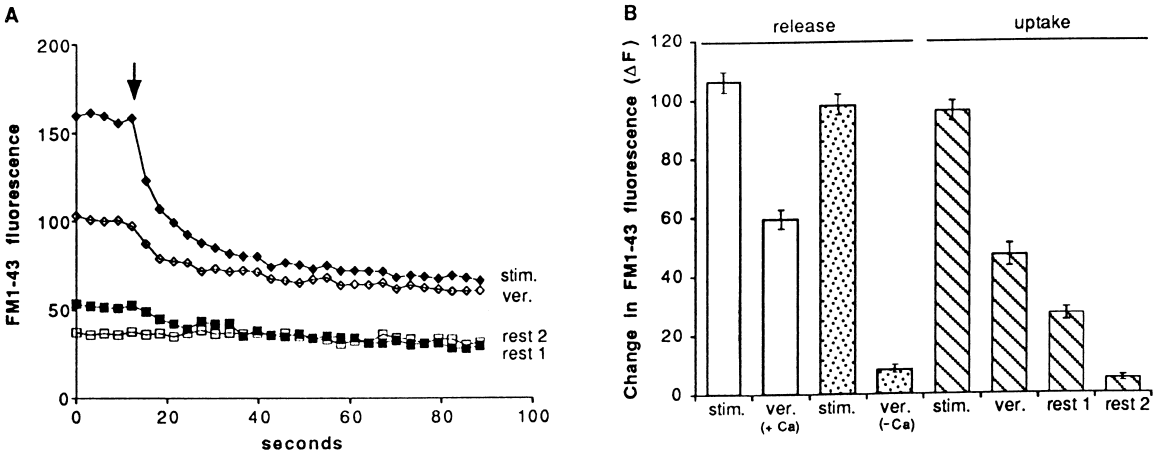

Figure 6. Uptake and Release of FM1-43 in Dendritic Boutons

(A) Uptake of FM1-43 (20 μM) in boutons of resting cells without (rest) or with veratridine (ver. 5 μM), and in electrically stimulated (stim.) cells; the quantity of dye uptake into boutons was measured by the fluorescence changes (curves) that occurred during identical periods of stimulation following the respective loading periods; onset of stimulation is indicated by the arrow; the curves are averages of 12 identical boutons in the same culture dish; the two curves obtained from resting cells without veratridine exposure show two populations of boutons that did (rest 1; closed squares) or did not (rest 2; open squares) take up dye that could be released by electrical stimulation.

(B) Summary of release experiments such as those illustrated in Figure 5, and of the uptake experiments described above. ΔF is the amount of dye specifically taken up by and, during electrical stimulation, released from individual boutons under the various conditions. Open columns represent ΔF during stimulation (stim.) and after a 2 min veratridine (ver.) exposure in Ca^{2+}-containing (+Ca) solution (means ± SEM of 56 boutons); dotted columns show ΔF during stimulation in Ca^{2+}-containing solution and the veratridine effect in Ca^{2+}-free (−Ca) solution (30 boutons). Striped columns summarize uptake experiments (48 boutons) as described in (A).

that showed no specific localization in the dendrites. The increase in background staining between the periods is due to the repetitive loading of the dye.

A statistical summary of all FM1-43 uptake experiments (n = 5; 48 boutons) is shown in Figure 6B (striped columns). All differences between the various conditions are highly significant ($p < 0.0001$). The 2 min uptake of releasable dye into boutons of resting cells in the absence of veratridine (rest 1 in Figure 6B) was 28% of that of a full load achieved by electrical stimulation. If the 19 boutons (rest 2) that had not taken up releasable dye are added, the average labeling was only 18% of that of a full load (data not shown). During a 2 min exposure of resting cells to veratridine, 49% of the full load was reached. This is similar to the 56% fluorescence change by veratridine during the same time period in the release experiments (Figure 6B, open columns). These results show that about half of the vesicular pool turns over in 2 min in the presence of 5 μM veratridine. Assuming that the total vesicular pool in a bouton is randomly mixed and labeled (Ryan and Smith, 1995) and that, depending on the size of the boutons, the pool contains 200–500 vesicles (for noncultured CA1 cells, see Harris and Sultan, 1995), one can calculate the average rate of release in a nonstimulated bouton as 0.3–0.8 vesicles/s. Veratridine increased the rate of release to 0.8–2 vesicles/s.

Finally, we have addressed the question whether small changes in Na^+ load by veratridine could influence the initial rate of exocytosis during excitation. For this purpose, boutons were labeled twice with FM1-43 by electrical stimulation (20 Hz for 20 s, followed by 80 s rest). After labeling and rinsing, the dye was unloaded during electrical stimulation (Figure 7). The dye was first released under control conditions in normal solution (closed squares). After the second loading, the cells were preincubated for 2 min in a solution containing 0.5 μM veratridine. This low alkaloid concentration did not depolarize the cells sufficiently to make them inexcitable. Therefore, the dye could be subsequently released

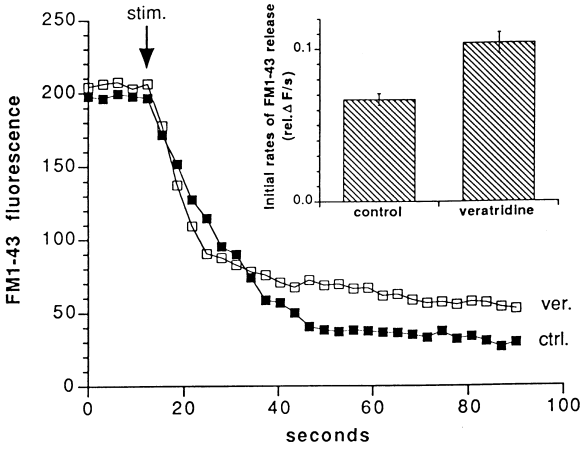

Figure 7. The Initial Rate of Evoked Exocytosis Is Enhanced by Veratridine

The curves show the release of FM1-43 from a single bouton during electrical stimulation; the bouton was loaded twice with the dye; the dye was unloaded in the absence (ctrl.) and presence (ver.) of veratridine (0.5 μM), the latter after a 2 min preincubation with the drug. The difference in ΔF between the two traces is 8% and within the normal variation between two loading and stimulation periods. The inset shows the initial rates of dye release from 29 identical boutons under control conditions and in the presence of veratridine.

in this solution by electrical stimulation (open squares). The initial rate of dye release from a single bouton depicted in the two curves in Figure 7 is enhanced by veratridine. The inset shows statistical results from 29 identical boutons measured under both conditions. The initial rate of FM1-43 release during electrical stimulation was increased by (0.5 μM) veratridine from 0.067 $\pm$ 0.004/s to 0.104 $\pm$ 0.007/s ($p < 0.0001$). These results show that already very small Na^+ loads in dendrites can cause an increase in the rate of evoked exocytosis.

Discussion

In this paper, we have shown that a Na^+ load in dendrites by veratridine causes an increased neurotransmitter release from nerve terminals, measured as sEPSCs, and an increased turnover rate of the vesicular pool in individual boutons, measured by the fluorescent dye FM1-43. Both effects of veratridine required the presence of $[Ca^{2+}]_o$, but were independent of Ca^{2+} channel openings. The quantal size was not changed in the sEPSC measurements, pointing to a presynaptic site of regulation of the increased sEPSC frequency by the Na^+ load. This has been directly demonstrated by the accelerated turnover rate of the vesicular pool in functional presynaptic boutons. There were boutons where no uptake of releasable dye could be measured during a 2 min period at rest, indicating a low probability of vesicle cycling (Ryan et al., 1996). However, like those with a higher spontaneous vesicle turnover, these boutons also became more active by veratridine exposure and by electrical stimulation. Veratridine-induced changes in $[Ca^{2+}]_i$ and in transmitter release were prevented by TTX and enhanced by ouabain, thus providing strong evidence that intracellular Na^+ accumulation is essential for both effects. An increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ by veratridine has been directly shown in dendrites (Figure 4). A highly probable link between Na^+ load and Ca^{2+} uptake is the Na^+/Ca^{2+} exchanger. Unfortunately, no specific inhibitor exists to provide definite proof for this link. However, the existence of this transport system in hippocampal neurons has been shown at the molecular level (Furman et al., 1993) and by immunocytochemistry (Reuter and Porzig, 1995; Juhaszova et al., 1996). The latter method provided evidence for particularly high densities of the exchanger in presynaptic boutons, but specific antibody staining could also be seen in dendrites.

Our previous functional studies have provided evidence that the exchanger contributes to the reduction of $[Ca^{2+}]_i$ after excitation in dendritic boutons of hippocampal cells (Reuter and Porzig, 1995). The present results suggest that the exchanger may be involved in intracellular Ca^{2+} accumulation if $[Na^+]_i$ is increased. This could be due to inhibition of Ca^{2+} extrusion or to a reversed mode of operation of the exchanger. A reversal of Na^+/Ca^{2+} exchange has been shown in numerous experiments in many cell types (for recent reviews, see Hilgemann et al., 1996). Since the exchanger is electrogenic (3 Na^+ for 1 Ca^{2+}), the driving force ($\Delta\mu_{Na/Ca}$) for Ca^{2+} efflux or influx is given by $\Delta\mu_{Na/Ca} = V_m - 3E_{Na} + 2E_{Ca}$ (Reeves, 1985), where V_m is the membrane potential and E_{Na} and E_{Ca} are the equilibrium potentials for the distribution of Na^+ and Ca^{2+}. A negative $\Delta\mu_{Na/Ca}$ means that Ca^{2+} is driven out of the cell, and a positive one means that Ca^{2+} uptake is favored. The absolute concentrations of $[Na^+]_i$ and $[Ca^{2+}]_i$ in boutons are unknown. However, the following calculations may emphasize the physiological importance of changes in $[Na^+]_i$ (see also Blaustein, 1988). With $[Na^+]_o$, $[Ca^{2+}]_o$, and V_m as reported in this paper (135 mM, 2 mM, and -51 mV) and assuming $[Na^+]_i$ and $[Ca^{2+}]_i$ to be 7 mM and 0.1 μM, respectively, one can calculate $\Delta\mu_{Na/Ca}$ as -25 mV, which means that Ca^{2+} is extruded. An increase in $[Na^+]_i$ by 5 mM would reverse $\Delta\mu_{Na/Ca}$ to $+16$ mV if the other factors remain constant, thus favoring Ca^{2+} uptake through the exchanger. In the small volumes of presynaptic boutons (0.12–0.43 μm^3; Harris and Sultan, 1995), such changes in $[Na^+]_i$ may well occur during excitation. Rapid Na^+ and Ca^{2+} entries following single action potentials as well as trains of spikes in dendrites of hippocampal neurons have previously been shown by SBFI and fura-2 measurements (Jaffe et al., 1992). Our results (Figure 4) are in essential agreement with this report. Moreover, the results shown in Figure 7 support the possibility that loads of $[Na^+]_i$ can acelerate exocytosis during such trains of action potentials.

The physiological importance of variation in $[Na^+]_i$ on synaptic transmission in hippocampal neurons, however, remains to be further elucidated. We have previously demonstrated an important role of the Na^+/Ca^{2+} exchanger in reducing $[Ca^{2+}]_i$ after trains of action potentials (Reuter and Porzig, 1995). This paper suggests that elevation of $[Na^+]_i$ increases $[Ca^{2+}]_i$, either by reducing the driving force for Ca^{2+} extrusion by the exchanger and/or by reversing the direction of the exchange. Direct evidence for an involvement of Na^+/Ca^{2+} exchange in transmitter release has been obtained in cultured retinal amacrine cells by Gleason et al. (1994). These investigators have made simultaneous electrophysiological recordings from pre- and postsynaptic pairs of these cells. They have identified a presynaptic membrane current as Na^+/Ca^{2+} exchange current that is involved in GABA release. The release was measured postsynaptically as inhibitory postsynaptic currents (IPSCs). It is interesting to note that in cultured mouse striatal neurons veratrine also evoked an increased Na^+ influx and spontaneous IPSCs resulting from GABA release (Fraser et al., 1993).

There are other reports that may imply that an elevation of $[Na^+]_i$ could have important physiological consequences for dendritic function. Action potentials are retrogradely propagated from cell bodies into dendrites (Markram et al., 1995; Larkum et al., 1996). Corresponding changes of $[Ca^{2+}]_i$ during trains of action potentials are more marked in the dendrites than in somata (Larkum et al., 1996). Although the change in $[Ca^{2+}]_i$ is rapid and most probably results from openings of voltage-gated Ca^{2+} channels, the decay of the fluorescence change is relatively slow and may take seconds (Larkum et al., 1996). It could be shown that a release of Ca^{2+} from intracellular stores does not affect the decay (Markram et al., 1995). However, it is quite possible that Ca^{2+} influx resulting from the Na^+ load during trains of action potentials contributes to the slow decay of $[Ca^{2+}]_i$ and, as shown in crayfish neuromuscular junction (Mulkey and Zucker, 1992), to posttetanic potentiation.

In conclusion, our results provide strong evidence that changes in $[Na^+]_i$ can modulate synaptic transmission and that the locus for this modulation is presynaptic. Ca^{2+} is essential for this process, and Na^+/Ca^{2+} exchange is a probable link. The physiological importance of this regulation needs further clarification.

Experimental Procedures

Hippocampal Cell Culture

Methods for dissociation of hippocampal neurons from 3- to 5-day-old Sprague–Dawley rats were similar to those described by Malgaroli and Tsien (1992) and by Ryan et al. (1993). In brief, cells from CA1–CA3 regions of the hippocampus were obtained by incubating small sections of this brain area in a digestion solution containing 3.4 mg/ml trypsin type XI (Sigma) and 0.9 mg/ml DNase type IV (Sigma). The cells were dissociated by gentle mechanical trituration in Ca^{2+}-free Hanks' solution supplemented with 12 mM $MgSO_4$, 0.4 mg/ml DNase, and 3 mg/ml bovine serum albumin. After centrifugation (80 × g), cells were plated on poly-L-ornithine coated coverslips at a density of about 30,000 within an area confined by a cloning cylinder (diameter 5 mm) glued to the coverslip by means of silicon grease (Dow Corning). Minimal essential media (GIBCO or Sigma) were supplemented with 29.2 mg/l glutamax I (Fluka), 6000 mg/l glucose, 25 mg/l insulin, 100 mg/l transferrin (Calbiochem), 5 mg/l gentamycin (Boehringer), and 10% fetal calf serum (GIBCO). Cultures were maintained at 37°C in the incubator, and media were changed every 2–3 days. On day 2, cytosine β-D-arabinofuranoside (ARA-C, 3 μM) was usually added to inhibit astrocyte growth, fetal calf serum was reduced to 5%, and 2% B-27 supplement (GIBCO) was also added to the medium.

Electrophysiology

For electrophysiological experiments, cultured hippocampal cells were used 5–14 days after plating. Membrane potentials and membrane currents were recorded by the patch-clamp method in the whole-cell configuration (Hamill et al., 1981; EPC7 amplifier, List Electronic, Darmstadt, Federal Republic of Germany). Patch pipettes contained the following solution: 135 mM Cs-gluconate, 2 mM $MgCl_2$, 0.1–5 mM EGTA, 5 mM Na_2-ATP, 0.2 mM GTP, 10 mM HEPES (buffered to pH 7.2 with CsOH). In some experiments, CsF or K-gluconate was used instead of Cs-gluconate. For measurements of sEPSCs, the membrane potential was usually clamped at −50 mV. Currents were recorded at sampling rates of 10–20 kHz and filtered at 1–2 kHz. On-line recordings were made with a Pentium 75 MHz computer driven by the CED software (version 6.0, Cambridge Electronics). For the analysis of sEPSCs, a fully automated software provided by Dr. A. Dityatev (Institute of Physiology, University of Bern, Switzerland) was used. The program is based on an algorithm by Ankri et al. (1994) and reliably detects and measures randomly occurring sEPSCs, providing data on current amplitudes and rise times (10%–90% of full amplitude), half width of each current, and intervals between two consecutive events. These data are the basis for the construction of sEPSC frequency versus amplitude histograms.

Voltage-gated I_{Na} and I_{Ba} were measured from holding potentials of −70 mV. For I_{Na} measurement, the membrane potential was hyperpolarized to −100 mV for 100 ms before each test pulse. I_{Na} and I_{Ba} were filtered at 4 kHz and 1 kHz, respectively.

Optical Measurements

Hippocampal cell cultures were used 14–22 days after plating. Coverslips were glued with silicon grease on a perspex chamber (with a volume of 0.3 ml) that permitted constant superfusion of the cultures with saline. Platinum electrodes were inserted into the solution. The chamber was placed on the stage of an inverted microscope (Axiovert 100) that is part of a confocal Laser Scan Microscope (LSM 410, Zeiss AG, Federal Republic of Germany). Specimens were viewed with a 40 × 1.3 NA oil immersion objective (Plan-Neofluar, Zeiss AG). They were illuminated through the objective with a 488 nm argon laser. Epifluorescence of FM1-43 was measured through a 515 nm long pass barrier filter and that of fluo-3 (peak fluorescence at 530 nm) through a 515–560 nm filter, both with the pinhole about 10% open. Fluorescence and differential interference contrast (DIC) pictures were displayed in a split configuration as 512 × 512 pixel images on the screen of a CPU 80 486/33 MHz computer. Time-lapse sequences were usually recorded at scanning rates of 2 s per image. Data were stored on the computer hard disc (320 MB) and later on transferred on a rewritable optical disc (128 MB).

The fluorescent styryl membrane probe FM1-43 (10–20 μM) (Betz et al., 1992) was added to the saline superfusing the cultured cells for 2 min. During this period, the cells were either left at rest, in the absence and presence of veratridine, or they were stimulated for 30 s at the beginning of the dye exposure. This was followed by washing of the cells in normal saline for 3–5 min. To measure changes in $[Ca^{2+}]_i$, the cells were incubated for 30–60 min in saline containing 10 μM fluo-3-AM. The pH indicator BCECF-AM (10 μM) was measured at the same settings as fluo-3-AM. For $[Na^+]_i$ measurement, cells were loaded up to 4 hr with SBFI-AM (15 μM) (Minta and Tsien, 1989; Fraser et al., 1993), and ratiometric measurements were made at 340/380 nm excitation and 520–560 nm emission. For these measurements, an imaging system video camera attached to an image intensifier (Videoscope International, Washington, DC) was used (Reber and Reuter, 1991). Fluorescence analyses were made from data stored on optical discs. All fluorescence changes during stimulation or during veratridine application could be prevented by the addition of TTX (1 μM). Functional synapses were identified by their release of FM1-43 during electrical stimulation. ROI were defined as bright fluorescence areas (<1.5 μm^2) after dye uptake. Changes in fluorescence intensity in ROIs before, during, and after stimulation were stored and plotted. When appropriate, data are presented as means ± SD or SEM. Significances of differences were calculated by paired or unpaired t tests.

Solutions and Materials

The saline superfusing the cells during the experiments had the following composition: 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (buffered to pH 7.4), 30 mM glucose. Flow rates were adjusted between 0.1 and 3 ml/s. All experiments were done at room temperature (~22°C). To block Ca^{2+} channels, in some experiments $CdCl_2$ (100 μM) or a combination of ω-conotoxin GVIA (2 μM), ω-agatoxin IVA (1 μM), and isradipine (2 μM) were added. In most experiments, the AMPA receptor blocker CNQX (10 μM) and in some experiments also the NMDA receptor blocker D-AP5 (25–100 μM) (both from RBI, Natick, MA) were added to the solution. Veratridine (Sigma) was used at concentrations of 0.1–50 μM and ouabain (Boehringer Mannheim) at 50 μM. The fluorescent probes FM1-43, fluo-3-AM, SBFI-AM, and BCECF-AM were obtained from Molecular Probes.

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